The Inhibition of Microsomal Triglyceride Transfer Protein through the use of Long dsRNA in Drosophila Melanogaster S2 Cells

Honors Project

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Table of Contents

List of Figures

INTERFERENCE OF MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN THROUGH THE USE OF RNAi IN SCHNEIDER'S S2 DROSOPHILA MELANOGASTER CELL LINE

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A major source of crop destruction worldwide is the pests, particularly insects, which consume them. As such, the overarching goal of our research is to explore the potential use of RNA interference (RNAi) in developing species-targeted insecticides. In our experiments, we use Drosophila melanogaster as a model for all insects, which share many similarities in the lipid-transfer pathways, yet are divergent enough to allow for species specificity. The target of RNAi is a molecule in our experiments is a molecule that is part of the lipid-transport pathway. The bulk of lipid transport in the hemolymph of insects is facilitated by lipoproteins, which package and transport neutral lipids from dietary or stored sources to the peripheral tissues to be used in ATP generation. The lipoproteins we have focused on are apolipophorin II/I (apoLpII/I) containing particles, of which there are at least two main requirements for formation: apoLpII/I and microsomal transfer triglyceride protein (MTP). While the role of MTP in apoLpII/I containing lipoprotein biogenesis is not fully defined, it is suggested to have a similar role as mammalian MTP in promoting the acquisition and filling of apoLpII/I with lipids for transport through the hemolymph. In this work, we have explored the inhibition of MTP expression by RNA interference, with the ultimate goal of examining its effect on lipoprotein biogenesis and the phenotype this confers on whole flies. This project specifically involved the construction and screening of an anti-MTP RNA that was capable of specifically knocking down the expression of MTP mRNA by >95% in drosophila S2 cells.

INTRODUCTION

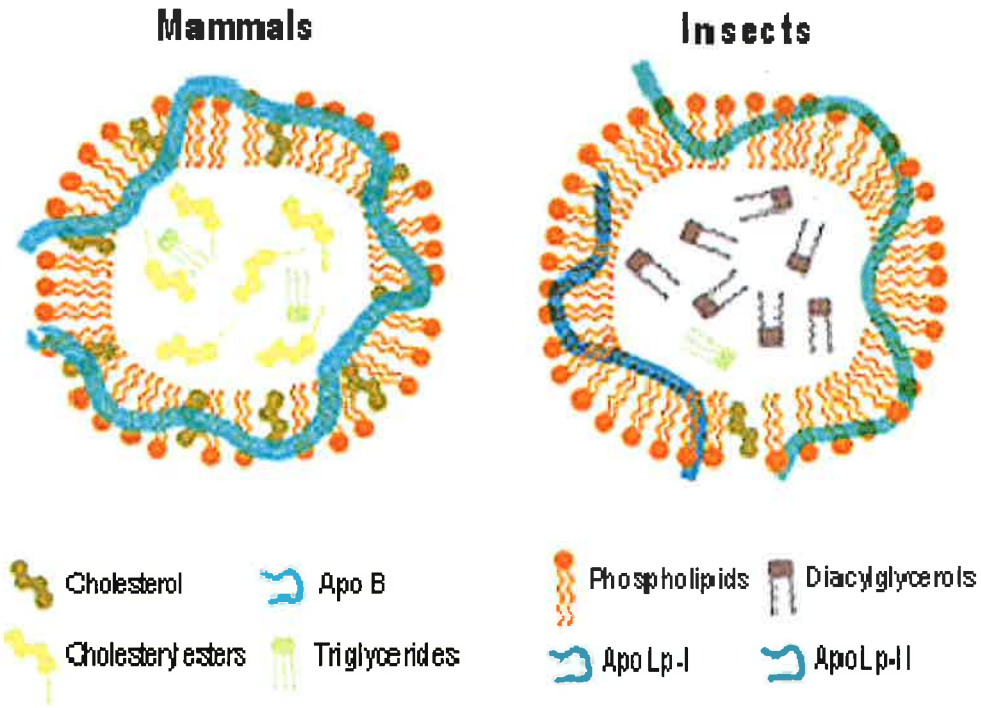
In both vertebrates and invertebrates, certain biological pathways are essential for homeostasis. Amongst these is the lipid-transfer pathway of which exists a group of genes known as the large lipid transfer protein (LLTP) gene family (2). The LLTP gene family include particles such as vitellogenins, apoLp-II/I, apoB and MTP and are involved in the biogenesis of various lipoproteins. Of these lipoproteins, our laboratory focuses on an insect form, apoLp-II/I containing lipoproteins, a close relative to apolipoprotein-B (apoB) containing lipoproteins in humans (1,2). When either apoLp-II/I or apoB associate with lipoproteins, they are known as apolipoproteins, which have the role of packagingand transporting neutral lipids such asdiglycerides, triglycerides and fatty acidsthrough the hemolymph (3).

Apolipoproteins are effective in the transport of lipids through the hemolymph due to their structure (Figure 1), which consists of a micelle shapedphospholipid membrane, either apoB in mammals or apoLp-II/I in insects which is intertwined with the phospholipids, and the hydrophobic neutral lipids trapped inside. The particular molecule of interestfor inhibition is MTP, the function of which is to transport neutral lipids from adipose or stored tissue to the apolipoprotein, which will then carry the lipids to their destination (1,2,3).

The method for which we will knock down MTP expression is by the use of RNA interference (RNAi). RNAi is based on short double stranded (ds) RNA that is complementary to the target mRNA (4). In insects, the mechanism for RNAi (Figure 2) begins with long dsRNA being inserted into the cells, whereupon an RNase, typically Dicer, cuts the long dsRNA into appropriate short interfering (si) RNA, which then forms

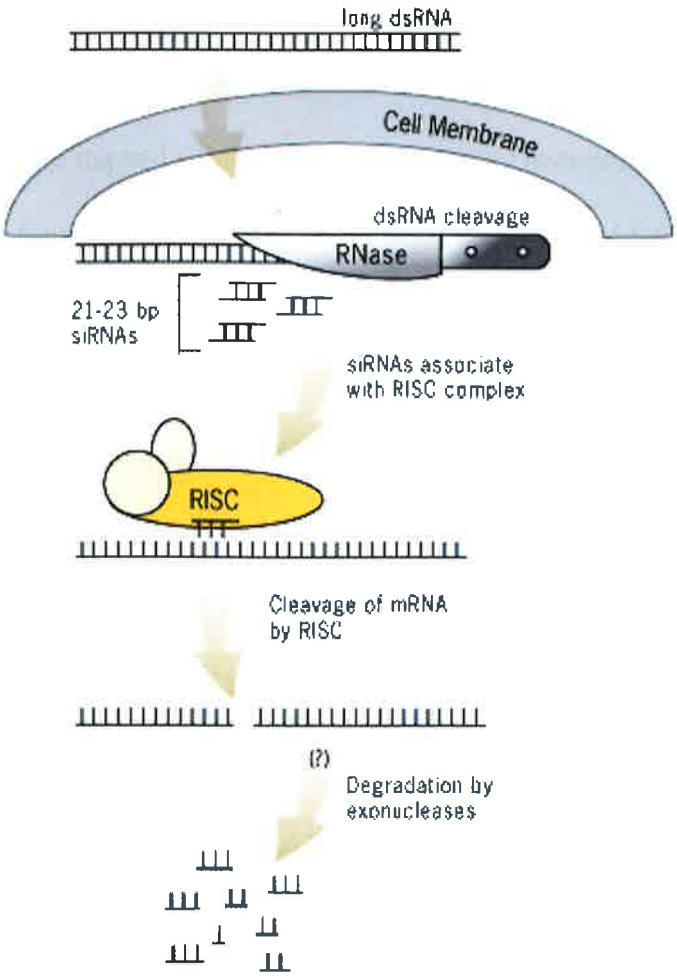
an association with the RISC complex (4). The RISC complextravels through the cytosol until it identifies an mRNA that is complimentary to the siRNA (4). When this locale is identified, the RISC complex anneals and cleaves the mRNA,effectively preventing the translation of a complete and functional protein (4). The use of long dsRNA is reserved for insects as it will illicit and antiviral response that will degrade the long dsRNA when introduced to either mammals or a mammalian cell line (5).

In our experiments, we create an effective anti-MTP long dsRNA in which we observed an approximate 50% diminution of MTP mRNA signal at 18μg of long dsRNA upon the initial attempt. Upon a subsequent titration with more potent long dsRNA, >95% diminution of signal was present at even the lowest dose of treatment, 1.5μg. Since MTP is essential for the production of apoLp-II/I containing LDLp, the disruption of MTP activity would effectively disrupt lipid transfer and consequently homeostasis in whole flies. As such, the success of the anti-MTP dsRNA insinuates that there is a possibility for a genetic pesticide using RNAi as its basis.



http://www.electronmicroscopy.nl/people/topics/lipophorins.html

Figure 1- An illustration of the basic structure of ApoB containing lipoprotein (Mammals) and ApoLp-II/I containing lipoprotein (Insects).



http://ambion.com/techlib/tn/115/11.html

Figure 2- This is the mechanism for RNAi with the starting reagent of long dsRNA.

MATERIALS AND METHODS

Thawing and Maintaining the Cell Line- Cells that were either shipped frozen or previously frozen were thawed using a protocol from the Drosophila Genomics Resource Center. Cells were transposed into a T-25 flask containing 5mL of Drosophila S2 growth media. Once the confluenceof the cells reached approximately 80%, the cells were transferred to a T-75 flask. The cells were then passaged approximately twice a week or whenever confluence reached above 85%. During the passaging process, all but 1mL of media was taken out and used to knock cells off of the sides of the T-75. The extra cells and media werediscarded and new media equivalent to the amount discarded away was replaced.

Determining the Correct Sequence of MTP for the Region for RNAi- The correct sequence of our MTP was determined by using specially designed oligonucleotides to amplify the amount of MTP sequence, 5' GCTTTAAATTGGGCATCTAC3' being the forward primer binding both to NM_136231 and SD01502 cDNA, and 5' TAGAAATATAGTTTTTATGC 3' being the reverse sequence also built to bind both to NM_136231 and SD01502 cDNA. The resulting PCR product was sent to Clemson University's sequencing lab for sequencing. Once the results of the sequencing were received, the derived sequence was aligned with the two sequences found in NCBI's database to determine which of the two was correct.

Preparing the Template for RNAi- Once the portion of MTP had been chosen, oligonucleotides were developed to be complimentary to the MTP gene and provide a PCR product to be used as a template for the *in vitro* transcription of the anti-MTP long dsRNA. The forward primer, 5' TAATACGACTCACTATAGGGGATGTGAAGAGTTAAAGCTACC 3',included 22

nucleotides of complementary sequence with an engineered T7 promoter appended to the 5' endto promote the future *in vitro* transcription. The reverse primer, 5' TAATACGACTCACTATAGGGTTACGGAACACGCAGAAG 3', included 18 nucleotides of complementary sequence with the same engineered T7 promoter as the forward primer appended to the 5' end. The complementary nucleotide portions of our oligonucleotideswere blasted against the *D. melanogaster* genome to verify their specificity, effectively preventing unwanted amplification of off-target products. A PCR consisting of 25 cycles of 94°C for 1 minute, 55°C for 1 minute and 68°C for 2 minutes with an 8 minute 68°C extension cycle at the end was used to amplify the amount of MTP RNAi template.

Preparing the anti-MTP long dsRNA- The anti-MTP long dsRNA was created using an *in vitro* transcription kit from MEGAscript. 2μl each of 10X T7 buffer, ATP, CTP, GTP, UTP, and T7 enzyme mix were added to 8μl (approximately 1μg) of the RNAi template upon which the reaction was incubated for approximately 16 hours at 37°C. The mixture was then introduced to 21μl of Nuclease-free water, 5μl 10X Digestion Buffer, 2μl DNase I, and 2μl RNase followed by a one hour incubation at 37°C. Added to this were 50μl 10X Binding Buffer, 150μl Nuclease-free water, and 250μl 100% ethanol. The new mixture was put on a column and centrifuged at maximum speed, 13,200xg, for 2 minutes. The flow-through was discarded and 500μl of wash solution was added and then centrifuged as before. The wash step was repeated a second time but with an extra 30 seconds of centrifugation added to completely dry the column. 50μl of elution solution was then added to the column and incubated in a 65°C for two minutes after which the column was centrifuged at maximum speed for 2 minutes. The

elution step was repeated a second time before the long dsRNA was checked via photospectrometry to determine both purity and concentration.

Applying the Construct to the Cells- Cells from our cultured stock were transferred into 6 well dishes with 4mL of Drosophila S2 growth media with approximately 9% FBS. Once the cells reached approximately 60% confluent, the FBS containing media was withdrawn and replaced with 1mL of serum free media. The long dsRNA was then added, in varying dosages, to the cells and incubated at room temperature for 30 minutes, after which, the serum free media was diluted with 3mL of the ~9% FBS containing media. The long dsRNA was then incubated at room temperature for 3 days before the cells were analyzed.

Extracting the RNA from the Cells- The extraction of RNAemployed a proprietary reagent called TRIzol. Media from the bathing cells was removed and TRIzol 2.25mlwas added. 200µl of chloroform was then added to the reaction and was followed by 15 seconds of before being subjected to centrifugation at 13,200xg for 15 minutes and at 4°C. The colorless phase was then transferred to a new tube upon which 500µl of isopropyl alcohol was added. After this, it was incubated at room temperature for 10 minutes and then centrifuged for 9,400xg for 10 minutes at 4°C. The RNA was then washed with 1ml of 75% ethanol. Then, the sample was vortexed and centrifuged at 7,400xg for 5 minutes. The sample was then dried via speedvac and redissolved in nuclease free water.

Determining the Knock Down Rate of the Construct-The purified RNA was subjected to RT-PCR utilizingthe same oligonucleotide primers that were used in the determination of the correct sequence. The PCR reaction was then subjected to gel

electrophoresis and the amount of knockdown was quantified based on the diminution of brightness of the bands.

RESULTS

Alignment of SD01502 cDNA with NM_136231- The original two sequences found in NCBI's database were aligned to determine the if there were any differences (Figure 3). Between the two sequences, there were eight different discrepancies, hence our cells and flies required sequencing to determine which of the two were correct in our lab.

Alignment of SD01502 cDNA and NM_136231 with the Sequence from our cells and flies- The two original sequences were aligned with our derived sequence (Figure 4) to determine which of the two in the database were correct. Our derived sequence corresponded with two of the areas of discrepancy and was homologous with the top sequence, SD01502 cDNA. Therefore, the SD01502 cDNA was the sequence used to design the long dsRNA.

*Anti-MTP long dsRNA is effective against MTP while being specific enough not to hit unrelated genes-*An RT-PCR of the total mRNA was performed with primers for both MTP and for actin (Figure 5 b,c). The negative control (Figure 5a) was run without reverse transcriptase to verify that there was no DNA contamination. No bands were present signifying that there was no DNA contamination present. The actin (Figure 5c) showed no sign of diminution in signal despite the changes in the amount of long dsRNA applied to the cells indicating that it had no effect on the actin encoding mRNA. The MTP treated with long dsRNA (Figure 5b) shows approximately 50% diminution in signal for both 18μg and 50μg of long dsRNA, leading us to believe that it is effective at much lower dosages. Similarly to actin,

apoLp-II/I (not pictured) saw no diminution of signal regardless of change in dosage.

Anti-MTP long dsRNA is effective against MTP at extremely low dosages- The total reaction was run again as a titration since 18μg and 50μg showed equal efficacy (Figure 6). The titration ranged from 1.5μg to 25μg of long dsRNA with intermediates that approximately doubled per sample. The band of interest (top band) disappears at even 1.5μg of long dsRNA meaning that there was >95% knock down at this dosage. As such, it can be concluded that the anti-MTP long dsRNA is effective at even small dosages.

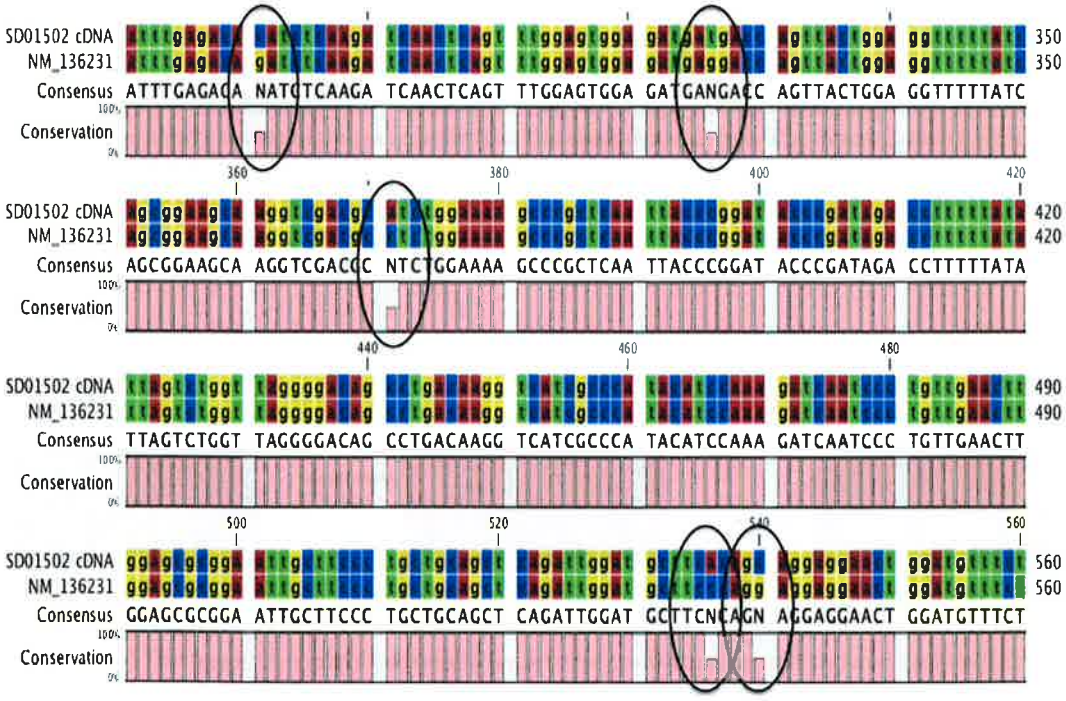


Figure 3- Alignment of two sequences of MTP found on NCBI's genomic database showing 5 of 8 total discrepancies. This alignment was done with CLC sequence viewer, a genomic and proteomic sequence alignment freeware.

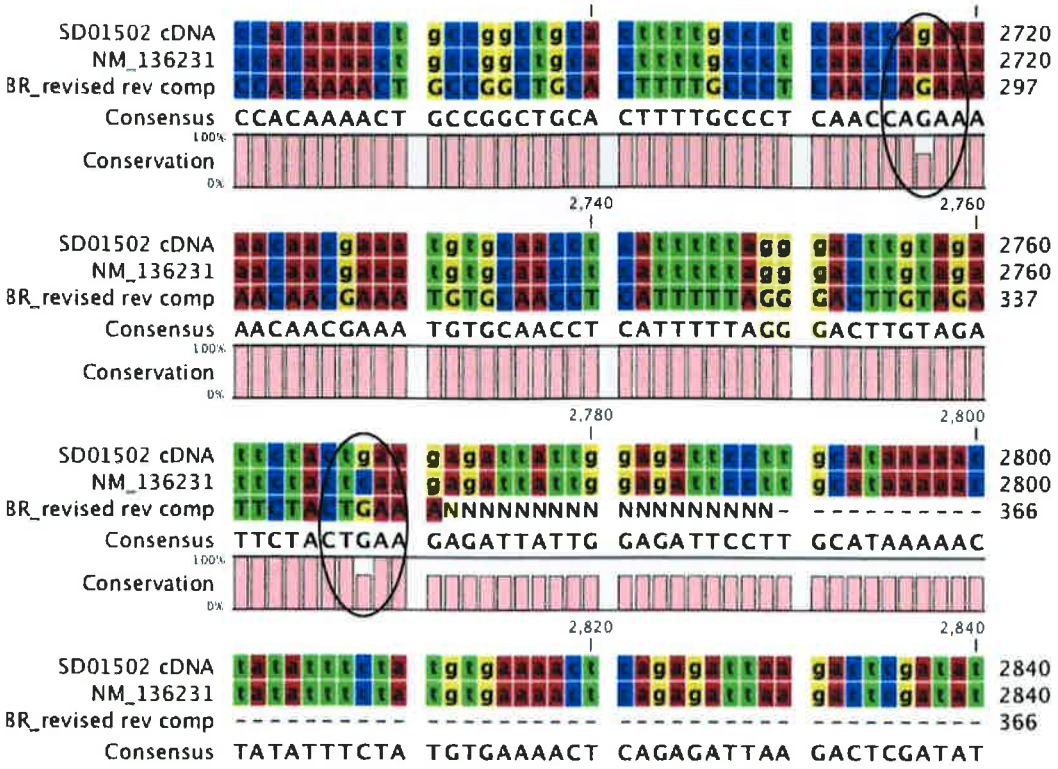


Figure 4- This is the alignment of the original two sequences, SD01502 cDNA as the top sequence, NM_136231 as the middle sequence and BR_revised rev comp (the corrected sequence received from Clemson University's sequencing laboratory) as the bottom sequence. The discrepancies are shown in the black ellipsis.

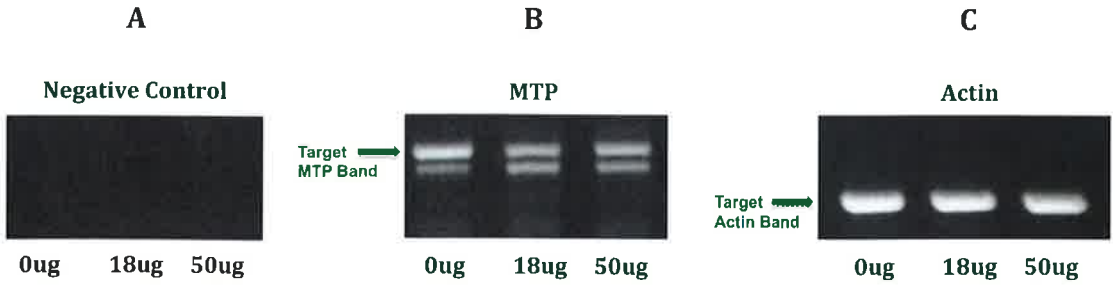


Figure 5- A) The negative control contained no reverse transcriptase and showed no bands under all three dosages (0μg, 18μg, and 50μg respectively). B) The MTP mRNA expression after adding the varying concentration of long dsRNA. The top band is the band of interest showing a bit of diminution of vibrancy at even 18μg of dsRNA. The bottom band is an artifact band. C) Actin treated with long dsRNA at the same concentrations. No diminution of signal was apparent.

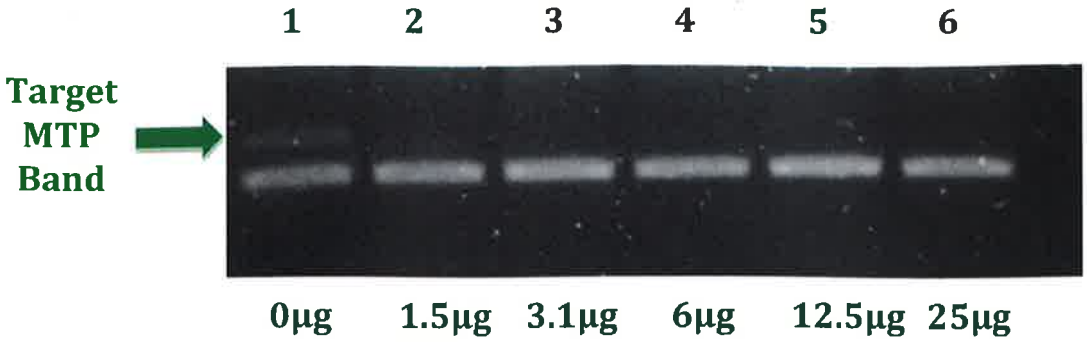


Figure 6- Titration of long dsRNA treatment targeting MTP in S2 cells using varying dosages (0µg, 1.5µg, 3.1µg, 6µg, 12.5µg, and 25µg respectively). The top band is the band of interest and like Figure 3, the bottom band is the same artifact band. This gel shows no signs of MTP mRNA signal after in lanes 2-6.

DISCUSSION / CONCLUSION

In our initial research, it was discovered that two genetic sequences for *D. melanogaster* MTP were present in the NCBI genomic database, SD01502 cDNA and NM_136231. When the two were aligned, many discrepancies (Figure 1) were identified. As the mechanism for RNAi requires specificity, our laboratory's cells and flies were subjected to genetic sequencing via Clemson University's sequencing lab. Upon receiving the electropherogram, the derived sequence was checked for miscalls followed by being aligned with the original two sequences (Figure 2), demonstrating that our sequence shared homology with SD01502 cDNA instead of NM_136231. We plan to share this information with NCBI so that any corrections that need to be made to the database are made.

Once we had identified the correct sequence, we developed and produced via an in house *in vitro* transcription (MEGAscript) our anti-MTP long dsRNA. We have determined that the anti-MTP dsRNA is effective and specific as it neither a similar gene (ApoLp-II/I) nor an unrelated gene (actin) was affected. Subsequently, we demonstrated, via titrated dosages, that our dsRNA was effective even at very low dosages.

We have not, as of yet, been able to identify the artifact bands seen in the figures 5 and 6, but we plan to conduct the experiment again to ensure that no mistakes were made. Once we have verified our experiments, our intentions are to develop a method to be used on whole flies, the theory remaining that a disruption of MTP biogenesis will result in a disruption of the homeostasis of whole flies. Considering that MTP is found in all insects thus far and that MTP is both similar enough to find in the genome yet

divergent enough that our long dsRNA can be species specific, the ability to develop a species specific genetic pesticide based on RNAi is well within reach.

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